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Lack of Effect of Feeding Lactoferrin on Intestinal Populations and Fecal Shedding of *Salmonella* Typhimurium in Experimentally-Infected Weaned Pigs

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ABSTRACT

Two experiments were conducted to evaluate the effect of the iron-binding molecule lactoferrin on reducing gut populations and fecal shedding of *Salmonella* Typhimurium in experimentally-infected weaned pigs. For each experiment, crossbred barrows and gilts were purchased locally and transported to our laboratory facilities. All pigs were fed a ground starter diet available for *ad libitum* consumption and randomly assigned to pen (2 pigs/pen) and treatment (10 pigs/treatment; 5 pens/treatment): Control [1.25 g whey protein concentrate (WPC)/kg BW (body weight)/d); 1X lactoferrin [0.25 g lactoferrin (LF) + 1.0 g WPC/kg BW/d]; and 5X LF (1.25 g LF/kg BW/d). Experimental treatments were fed prior to inoculation via oral gavage with *Salmonella* Typhimurium. Rectal swabs (collected daily for 4 days) for quantification of the challenge *Salmonella* strain and scour and activity scores, and body temperatures recorded daily following inoculation. Five days post-challenge, pigs were euthanized and tissue and luminal content samples aseptically collected from the stomach, ileum, cecum, spiral colon and rectum. Additional tissue samples were collected from the ileo-cecal lymph nodes, spleen, tonsil, and liver. Quantitative and qualitative bacterial culture was conducted for the challenge strain of *Salmonella*. No treatment differences ($P > 0.10$) were observed for daily fecal shedding or luminal concentrations of *Salmonella* in either experiment. The percentage of tissue samples *Salmonella* positive was not significantly different among treatments with the exception of liver tissue in Experiment I, which was lower ($P < 0.05$) in the 1X and 5X treatments compared to control pigs. Body weights and BW change were not affected ($P > 0.10$) by treatment. Following inoculation, body temperatures, scour and activity scores were not different when examined by day or when data was combined across days. Future research should evaluate increasing the duration of feeding and/or the levels of lactoferrin fed in conjunction with a more subtle *Salmonella* challenge.

Keywords: *Salmonella*, lactoferrin, pigs

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INTRODUCTION

Salmonella is the second leading cause of foodborne illness, resulting in an estimated 1.4 million cases every year (Foley and Lynne, 2008). Of these human cases of salmonellosis, 6-9% are associated with the consumption of pork or pork products (Frenzen *et al.*, 1999). *Salmonella* has been isolated throughout all stages of the pork production cycle and has received considerable attention, not only from a food safety standpoint, but additionally, because *Salmonella* can cause clinical infection in swine. *Salmonella* positive pigs are thought to arise from one of two general factors, inputs (pigs, feed, rodents, etc.) and activities within the swine production process (mixing of animals, transport, housing, and other management factors). Early weaning (< 21 d of age) which has gained in popularity, results in an immature digestive tract (Shields *et al.*, 1980) and perhaps more importantly, a decrease in immune system function (Blecha *et al.*, 1983), both of which would favor *Salmonella* colonization in these animals. To respond to the challenge of providing a safe pork product for the consumer, improve swine health, and maintain a safe environment, the development of pre-harvest, "on-farm" intervention strategies is crucial.

Most all bacteria, including the pathogenic bacteria *Campylobacter*, *E. coli* and *Salmonella*, require iron for survival and important intracellular reactions (Naikare *et al.*, 2006; Brock, 1980; Ratledge and Dover, 2000), thus iron-sequestering compounds such as lactoferrin and transferrin provide a primary nonspecific host defense system against microbial infection. A variety of preventative and therapeutic strategies for treating bacterial infections are based upon interfering with microbial iron acquisition and utilization. The immune system likewise exploits the iron requirement of bacteria, utilizing iron withholding as an essential antimicrobial component of the innate immune system.

Lactoferrin is a major iron-binding protein present in multiple body fluids and found in particularly high concentrations in both human and porcine milk (Gislason *et al.*, 1993; Vorland, 1999). The iron-binding abilities of lactoferrin enable it to scavenge iron within the intestinal tract thereby depriving microorganisms of this critical element and inhibiting their metabolic activities (Naidu *et al.*, 1993). Facilitating iron absorption, stimulation of mucosal differentiation, and modulation of mucosal immunity have been suggested as possible functions of lactoferrin within the gastrointestinal tract (Lonnerdal and Iyer, 1995). Additional research indicates that the antimicrobial properties of lactoferrin go beyond simple iron deprivation and include damage of the outer membrane and subsequent permeability alterations (Ellison *et al.*, 1988) and modulation of bacterial motility, aggregation and adhesion (Valenti and Antonini, 2005). Lactoferrin has been shown to inhibit growth of several important bacteria, including *Salmonella*, *E. coli*, *Listeria, Streptococcus* and *Shigella* (Weinberg, 1995; Lonnerdal and Iyer, 1995; Pakkanen and Aalto, 1997; Weinberg, 2001; Lee *et al.*, 2004). Other research has demonstrated that oral administration of lactoferrin decreases bacterial infections within the gastrointestinal tract while at the same time increasing populations of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* with low iron requirements (Petschow *et al.*, 1999; Weinberg, 2001; Tomita *et al.*, 2002; DiMario *et al.*, 2003; Teraguchi *et al.*, 2004; Sherman *et al.*, 2004). Thus, based on the antimicrobial activities of lactoferrin, the objective of the current project was to determine whether oral administration of lactoferrin would significantly reduce the populations of *Salmonella* within the gastrointestinal tract of experimentally-infected pigs.

MATERIALS AND METHODS

Experiment I

Forty crossbred barrows and gilts (avg. BW = 24 kg) were purchased locally and transported to our laboratory facilities. Upon arrival all pigs were weighed, eartagged and a rectal swab collected for culture of wild-type *Salmonella*. All pigs were housed in environmentally-controlled isolation rooms (10 pigs/room) for one week and maintained on a pelleted commericial pig starter feed available for *ad libitum* consumption. The following week, pigs were moved to another part of the same building and randomly assigned to pen (2 pigs/pen) where they remained for the remainder of the experimental period. Treatments (detailed below) were randomly assigned to pen, therefore a few pigs were moved to ensure similar sex and BW distribution among treatments. Two days following movement into the experimental pens, adaptation from the pelleted to a ground meal feed was initiated. All pigs were fed a 50/50 mix of pelleted and meal feed for four days, 25/75 pellets and meal for 2 days and 100% meal feed for three days prior to initiation of the experimental diets. One day prior to the start of the experiment, all pigs were weighed and given new eartags.

Experimental treatments (10 pigs/treatment; 5 pens/treatment) consisted of: Control [1.25 g whey protein concentrate (WPC)/kg BW/d); 1X lactoferrin [0.25 g lactoferrin (LF) + 1.0 g WPC/kg BW/d]; 5X LF (1.25 g LF/kg BW/d); and Non-infected Control (1.25 g WPC but not inoculated with *Salmonella*). Feed intakes were recorded and used to calculate an average daily feed intake per treatment. Based on the average feed intakes, diets were mixed to provide the amounts above of the experimental compounds per pig each day. Body weight and feed intake were recorded weekly and the feed adjusted accordingly. Experimental treatments were fed for a total of 20 days. On day 15 of the experimental diets, all pigs were inoculated via oral gavage with *Salmonella* Typhimurium (2.6×10^{10} in 20 mL TSB). Rectal swabs were collected daily for 4 days for quantification of the challenge *Salmonella* strain as described below. Scour and activity scores (for each pen) were recorded daily following inoculation through necropsy. Body temperature was recorded daily for each pig following inoculation using the ThermoFlash® electronic thermometer (PRO-IR ZH-36 Veterinary thermometer; Synergy USA, Miami, FL). Five days post-challenge, pigs were sedated with an intramuscular injection of a cocktail containing Ketaset, Telazol (Ft. Dodge Laboratories, Kansas City, MO) and Xylazine (Phoenix Scientific, St. Joseph, MO) prior to administration of a lethal dose of Euthasol (Delmarva Laboratories, Midlothian, VA). Tissue and luminal content samples were aseptically collected from the stomach, ileum, cecum, spiral colon and rectum. Additional tissue samples were collected from the ileo-cecal lymph nodes, spleen, tonsil, and liver. All tissue and content samples were cultured as described below immediately following collection. Non-infected control pigs were not euthanized for reasons discussed below.

Experiment II

A second experiment was conducted, similar to the first, with the exception that much younger pigs were utilized. Thirty crossbred piglets (average BW = 6.6 kg), were purchased within one week of weaning and transported to our laboratory facilities. Pigs were weighed, eartagged, rectally swabbed and randomly assigned to pen (2 pigs/pen). All pigs were provided a pig starter ration (ground) and water for *ad libitum* intake. Following analysis of initital BW, a few pigs were moved to assure equal distribution of BW among treatments. Animals were provided a 4 day adjustment period to acclimate to pens and diet and determine feed intakes. Following this acclimation, treatments were initiated (d 1) and administered throughout the remainder of the experimental period (13 total days; 10 pigs and 5 pens/treatment). Treatments were identical to those used in Experiment I with the exception that a non-infected control treatment was not included due to the ease in which pigs in this treatment acquired *Salmonella* in the first experiment. On day 8 of the experiment, all pigs were orally inoculated with 8 mL of TSB containing 5.6×10^{9} cfu *Salmonella* Typhimurium. Rectal swabs, body temperature, activity and scour scores were collected daily for 5 days following inoculation. All animals were euthanized and necropsied as described above on d 13. Body weights were recorded upon arrival and on d 1, 8 and 13 of the experimental period.

Bacterial Culture

<u>Experiment I</u>

Rectal swabs were collected using a foam-tipped swab (ITW Texwipe, Mahwah, NJ). Swabs taken prior to inoculation were incubated in 9 mL tetrathionate broth (37° C, 24 h), followed by a second enrichment [100 µL to 5 mL of Rappaport-Vassiliadis (RV) R10 broth; 42° C, 24 h], before spread plating on brilliant green agar (Oxoid Ltd., Hampshire, UK) containing novobiocin (BGA_{NOV}; 20 µg/mL) and novobiocin plus naladixic acid (BGA_{NN}; 20 and 25 µg/mL, respectively) for detection of any wild-type *Salmonella*. A few pigs were naturally-colonized with a wild-type *Salmonella* capable of growth on BGA_{NOV}, therefore all samples collected following inoculation of pigs were streaked on BGA_{NN}. The inoculation strain of *Salmonella* was enumerated in luminal contents by direct plating from a mixture of 1 g contents in 10 mL of tryptic soy broth (TSB) onto XLD agar using a commercially available spiral plater (Spiral Biotech Autoplate 4000; Advanced Instruments, Inc., Norwood, MA). Black colonies were counted following incubation (37° C, 24 hours). An additional 1 g of luminal content or tissue sample was enriched (qualitative culture) in 10 mL of tetrathionate broth, transferred to RV and plated as described above for the post-inoculation swabs. Following incubation at 37°C for 24 h, BGA_{NN} plates containing pink colonies exhibiting typical *Salmonella* morphology were considered positive.

Experiment II

Upon arrival all pigs were naturally-colonized with *Salmonella* capable of growth on BGA_{NOV} and BGA_{NN}, therefore fecal swabs were collected daily throughout the entire experimental period and plated on BGA_{NOV} to monitor shedding of the wild-type *Salmonella* and any response to experimental treatments. Due to the presence of this *Salmonella*, the inoculated strain of *Salmonella* Typhimurium was made resistant to rifampicin (25 µL/mL; prior to administration to the pigs) and all post-inoculation swabs and necropsy samples additionally plated on BGA_{NNR}. Spiral plating of luminal content samples was done on XLD + novobiocin and XLD + rifampicin. All enrichment procedures were identical to those used in Experiment I described above.

Statistical Analysis

All data were analyzed using SAS Version 9.1.3 (SAS Inst. Inc., Cary, NC, USA). Quantitative culture data from the luminal contents (log-transformed), body weight and temperature data were subjected to analysis of variance appropriate for a completely randomized design. Qualitative culture data (incidence of positive luminal content and tissue samples) was subjected to Chi-square analysis using the PROC FREQ procedure. Daily rectal swab culture results (positive or negative), activity and scour scores were analyzed using the PROC MIXED procedure for repeated measures with treatment, day and treatment x day interaction included in the model. For some samples, *Salmonella* was recovered only from enriched specimens or not at all indicating that concentrations were below our limit of detection (< 20 cfu/g of contents). Due to the inherent assumption that these samples were below the limit of detection (rather than assumed to be truly zero), we assigned a value of 1.0 cfu/g to all quantitative data prior to statistical analysis. Results were considered statistically significant at the 0.05 level for type-one error.

RESULTS

Experiment I

All pigs were pre-screened for *Salmonella* three times prior to initiation of the experimental diets using rectal swabs. The first and second collections were plated on BGA_{NOV} for detection of any wild-type *Salmonella*. All samples from the first collection were negative while five pigs were *Salmonella* positive in the second collection (serogroups B and C_2). The second and third collections were plated on BGA_{NN} to determine its suitability for detecting the challenge strain of *Salmonella* post-inoculation. All pigs were culture negative on this medium (data not shown).

Rectal swabs collected over the 4-d post-inoculation period were mostly positive in all treatment groups, including the non-infected control pigs. Di-

Table 1. Daily fecal shedding, luminal content populations of *Salmonella* (CFU/g $\log_{10}$) and *Salmonella* positive tissue and luminal content samples in pigs experimentally-infected with *Salmonella* Typhimurium and fed diets containing 1.25 g whey protein concentrate (WPC)/kg BW(body weight)/d = (Control); 0.25 g lactoferrin (LF) + 1.0 g WPC/kg BW/d (1X); 1.25 g LF/kg BW/d (5X); or 1.25 g WPC but not inoculated with *Salmonella* (NI Cont) – Experiment I.

	Treatment				
Item	Control	1 X	5 X	NI Cont	P > F
Rectal Swabs (% positive)[a]					
d 1	100	100	100	100	1
d 2	90	90	80	80	0.85
d 3	100	90	100	80	0.26
d 4	100	90	80	20	0.0002
Overall	97.5	92.5	90	70	0.001
Luminal contents					
Direct plate [cfu/g ($\log_{10}$)]					
Stomach	1	1	1	.	1
Ileum	2.4	3.4	2.7	.	0.31
Spiral colon	2.7	3.7	2.8	.	0.14
Cecum	3.2	3.7	3.2	.	0.23
Rectum	2.4	3.2	2.4	.	0.17
% positive after enrichment					
Stomach	50	40	60	.	0.67
Ileum	90	80	100	.	0.33
Spiral colon	90	80	70	.	0.54
Cecum	100	90	90	.	0.59
Rectum	60	80	50	.	0.37
Tissue					
% positive after enrichment					
Ileo-cecal lymph nodes	100	90	100	.	0.36
Spleen	10	30	20	.	0.54
Tonsil	80	80	70	.	0.83
Liver	70	10	30	.	0.02
Stomach	60	60	90	.	0.24
Ileum	80	100	90	.	0.33
Spiral colon	100	100	100	.	1
Cecum	90	90	90	.	1
Rectum	90	90	90	.	1

[a]By day post-inoculation

rect streaking of the swab onto the agar was conducted on d17 – 19 to get an indication of *Salmonella* concentrations in the feces. A positive swab via direct plating would be indicative of a higher concentration of *Salmonella* being shed by the animal, compared to a swab requiring enrichment to test culture positive. All direct swabs were negative in the non-infected control treatment, therefore only the direct plating data for the infected-control, 1X and 5X treatments were analyzed and no differences ($P > 0.10$) observed (data not shown). Following enrichment, no treatment differences ($P > 0.10$) were observed on each of the first three days post-inoculation, but by day 4 and when daily rectal swab data was combined and examined across days, non-infected controls had fewer *Salmonella* positive swabs ($P < 0.01$; Table 1). We certainly expected the non-infected controls to have a lower prevalence of *Salmonella*-positive fecal swabs throughout the experimental period and were surprised by the number of positive animals early on in the experiment. Although the non-infected control pigs were housed in the same room as infected-animals, they were not able to have any animal to animal contact. Obviously, contamination of these pigs could have occurred via workers, air-movement, or other factors, however, finding 100% of these pigs *Salmonella*-positive one day following inoculation of the other pigs, was not expected and highlights the ease in which *Salmonella* is transmitted among pigs and the short time duration required for fecal shedding following exposure. We did not serogroup any of the isolates from these animals to determine if the recovered *Salmonella* was the same as used to infect pigs in the other treatments as this information would be of limited value. Non-infected controls were included to determine if the whey-protein concentrate influenced growth, however, as all of these pigs were *Salmonella*-positive at some point in the experiment the decision was made not to necropsy this group.

Necropsy results are presented in Table 1. Concentrations of the challenge-strain of *Salmonella* were not statistically different among treatments throughout the GIT, although the 5X treatment had populations more similar to controls than did the 1X treatment, which had concentrations numerically higher in contents from the ileum, spiral colon, cecum and rectum. All stomach content samples were negative in all treatments. Following enrichment, luminal content samples were not different ($P > 0.10$) among treatments. Tissue samples were also not different ($P > 0.10$) among treatments, with the exception of liver tissue, which was lower ($P < 0.05$) in the 1X and 5X treatments compared to control pigs.

Body weights and BW change were not affected ($P > 0.10$) by treatment, although the 5X pigs gained 2.4 kg more than infected-control animals (data not shown). Following inoculation, body temperatures were not different when examined by day or when data was combined across days. A trend ($P < 0.10$) was observed on d 18 and when data was combined across days, however the differences were slight and do not suggest treatment effects (data not shown). There was not a significant treatment x day interaction for activity or scour scores ($P > 0.10$), nor were significant differences observed when data was combined across days (data not shown).

Experiment II

The majority of pigs were *Salmonella*-positive during the pre-screening process, therefore we attempted to examine the effect of the experimental treatments on the wild-type *Salmonella* strains as well as the experimentally-inoculated strain. Table 2 presents the prevalence of *Salmonella* positive rectal swabs (pre-challenge for the wild strain; post-challenge for all *Salmonella*) as well as necropsy results. To distinguish the two types of *Salmonella*, samples were plated on BGA_{NOV} for the wild-type *Salmonella* and BGA_{NNR} for *Salmonella* Typhimurium (challenge strain). No differences ($P > 0.10$) were observed in the prevalence of rectal swabs positive for the wild-type *Salmonella* following direct plating or after enrichment during the seven days of feeding the experimental diets pre-challenge. Post-challenge, no differences ($P > 0.10$) were observed for shedding of the wild-type strain (direct plated and enriched samples), while a trend ($P < 0.10$) was observed for the inoculated strain following direct plating (prevalence

Table 2. Daily fecal shedding, luminal content populations of *Salmonella* (CFU/g log) and *Salmonella* positive tissue and luminal content samples in pigs both naturally and experimentally-infected and fed diets containing 1.25 g whey protein concentrate (WPC)/kg BW (body weight)/d = (Control); 0.25 g lactoferrin (LF) + 1.0 g WPC/kg BW/d (1X); or 1.25 g LF/kg BW/d (5X). Naturally-occurring and experimentally-infected strains of *Salmonella* were plated on brilliant green agar containing novobiocin (nov) and novobiocin plus naladixic acid and rifampicin (nnr), respectively – Experiment II.

	Treatment							
	Control		1 X		5 X		P > F	
Item	nov	nnr	nov	nnr	nov	nnr	nov	nnr
Rectal swabs (% positive)								
Pre-challenge - overall (n=70/trt)								
Direct plate	7.1	.	5.7	.	2.9	.	0.51	.
Enriched	52.3	.	54.3	.	52.9	.	0.98	.
Post-challenge - overall (n=40/trt)								
Direct plate	27.5	25	40	50	32.5	40	0.49	0.07
Enriched	87.5	87.5	87.5	90	92.5	92.5	0.71	0.76
Luminal contents								
Concentration [cfu/g ($\log_{10}$)]								
Stomach	1	1	1	1	1	1	1	1
Ileum	1.2	1.2	1.4	1.1	1.8	1.7	0.37	0.14
Spiral colon	1.8	1.5	2.4	2.1	2.3	2.3	0.48	0.16
Cecum	1.9	1.6	1.4	1.3	1.8	1.6	0.54	0.75
Rectum	1.4	1.4	1.8	1.3	1.8	1.8	0.54	0.34
% positive w/enrichment								
Stomach	10	10	0	0	30	30	0.13	0.13
Ileum	60	60	80	80	80	80	0.51	0.51
Spiral colon	100	100	100	100	100	100	1	1
Cecum	100	100	70	70	90	90	0.13	0.13
Rectum	100	100	100	100	90	90	0.37	0.37
Tissue (% positive w/enrichment)								
Stomach	50	50	50	50	40	40	0.87	0.87
Ileum	90	90	90	90	90	90	1	1
Spiral colon	100	100	100	100	100	100	1	1
Cecum	80	80	100	100	100	100	0.18	0.18
Rectum	90	90	100	100	90	90	0.59	0.59
Ileo-cecal lymph nodes	60	60	90	90	70	70	0.3	0.3
Spleen	10	10	40	30	30	20	0.3	0.54
Tonsil	50	50	30	30	20	20	0.35	0.35
Liver	100	100	100	100	100	100	1	1

in 1X and 5X treatments numerically higher than control pigs). No differences were observed for *Salmonella* Typhimurium following enrichment of the rectal swabs. Concentrations of *Salmonella* in the luminal contents throughout the GIT were not different ($P > 0.10$) among treatments for either the wild-type or inoculated *Salmonella* strains, nor was prevalence different ($P > 0.10$) following enrichment of content samples. Similarly, the prevalence of positive tissue samples following enrichment were not different among treatments for either *Salmonella* type.

Body weights and BW change were similar ($P > 0.10$) among treatments throughout the experiment (data not shown). Similar to the first experiment, pigs in the 5X treatment exhibited a numerical increase in BW gain compared to control animals following inoculation with the challenge strain. No differences ($P > 0.10$) in body temperature were observed pre- or post-challenge, however there was a tendency ($P = 0.09$) for pigs in the 5X treatment to have higher temperatures than the control and 1X animals (data not shown). No treatment x day interactions were observed for activity or scour scores, therefore data was combined and presented as pre- and post-challenge and across all days. Neither activity nor scour scores were statistically different pre- or post-challenge or when data was combined across the entire experimental period (data not shown).

DISCUSSION

Oral administration of lactoferrin has been reported to provide host protection against various diseases in animals and humans, including infections, cancers and inflammations (Tomita *et al.*, 2002). Teraguchi and colleagues (2004) concluded that oral lactoferrin enhances the systemic or peripheral immune responses to pathogens, their components, as well as mucosal immunity in the intestines and that these responses may contribute to elimination of the pathogens and/or a reduction of the symptoms. Lactoferrin binds to *Salmonella* Typhimurium and bovine lactoferrin has been shown to have an iron-dependent bacteriostatic effect on this pathogen (Naidu *et al.*, 1993; Ochoa and Cleary, 2009). Both bovine and human lactoferrin inhibit the adherence and invasion of *Salmonella* to tissue culture cells (Bessler *et al.*, 2006). Wang and co-workers (2006) reported a beneficial effect of lactoferrin supplementation on growth performance of weaned piglets and concluded the use of lactoferrin to improve nonspecific immunity and strengthen host defenses would be a good method of protecting weaned pigs from infections and stress due to weaning. Taken together, we reasoned that administration of lactoferrin to pigs may reduce the gut populations and fecal shedding of *Salmonella*.

Due to some facility constraints and pig availability, the pigs in the first experiment were older and larger than we considered ideal for this experimentation. We hypothesized that lactoferrin treatment had the best chance of success in a younger animal with an immature or under-developed gut microbiota where *Salmonella* had less competition from other microbes and was therefore more likely to flourish. However, as pigs can be exposed to *Salmonella* at all stages of the pork production cycle, the decision was made to examine the effect of lactorferrin in the larger animals. Whether or not this was the reason for the lack of treatment effects in the first experiment is unknown. The percentage of positive rectal swabs and luminal contents were similar in the two experiments, indicating that the experimental challenges were similarly effective in the older and younger pigs and that at these ages, differences in the gut microbial ecosystem were negligible in terms of affecting the challenge strain of *Salmonella*.

The second experiment was conducted virtually identical to the first with the exception that we used much younger pigs and had the added bonus that the pigs were "naturally-colonized" with *Salmonella*. In theory, this should provide for a more realistic evaluation of the treatments, however to ensure all pigs were similarly infected, animals were also administered the challenge strain of *Salmonella* Typhimurium. No effects of treatment were observed on either the naturally-colonized or experimental strain of *Salmonella*.

The lack of any observable benefits due to the lactoferrin treatment in reducing *Salmonella* populations or the severity of infection in these experiments is disappointing but may be explained by one or a combination of factors discussed below. The most plausible explanation is that the challenge doses (10^9 and 10^{10} cfu *Salmonella*) utilized were such that they simply overwhelmed any beneficial effects provided by the lactoferrin. A lower dose, more realistic of what the pigs might be exposed to in a production setting, may have provided a better test for the lactoferrin treatments examined. However, in our experience with experimental inoculation, the lower doses are generally cleared quickly in any age animal except those with a very immature or disturbed gut microflora. As pigs in both experiments were weaned and eating well, we expected that a larger challenge would be necessary to establish *Salmonella* within the gut and produce concentrations that could subsequently be detected in the luminal contents and gut tissues at necropsy several days post-inoculation.

Similar to our research, Sarelli and co-workers (2003) evaluated lactoferrin for preventing *E. coli* diarrhea in weaned pigs. They reported no significant effect on occurrence of diarrhea, fecal *E. coli* counts, or weight gain in pigs dosed twice daily with lactoferrin. The authors hypothesized that continual feeding of the lactoferrin in the feed may provide more protection than the twice-daily dosing regimen they used and likewise suggested that the massive dose of *E. coli* administered to the pigs may have simply overwhelmed any protective effect exerted by the lactoferrin and that future research should employ challenges similar to what would be encountered by the pigs in commercial production settings. Contrary to these findings and our own reported herein, Lee and co-workers (1998) reported oral lactoferrin protected piglets against lethal shock induced by intravenously administered *E. coli* LPS (endotoxin) with significantly less mortality compared to the control treatment.

Others have reported a beneficial effect of lactoferrin and lactoperoxidase system (LP-s) on experimentally-induced *E. coli* diarrhea in calves with improvements in mortality, occurrence of severe diarrhea and duration of diarrhea observed (Still *et al.*, 1990). A combination of lactoferrin and LP-s given orally decreased *E. coli* counts in the intestine and feces of calves and likewise reduced the severity of diarrhea (van Leeuwen *et al.*, 2000). In the current research, diarrhea was observed in pigs during both experiments following *Salmonella* inoculation, but contrary to the research by Still and van Leeuwen, no beneficial effects of lactoferrin were observed on the incidence or severity of diarrhea.

A second explanation for the lack of a treatment effect in this research may be explained by the adaptations bacteria make in order to compete with iron-sequestering compounds such as lactoferrin. Some strains of bacteria adapt to the iron-deprived conditions by producing their own high affinity iron chelators called siderophores, which compete directly with lactoferrin for iron (Crosa, 1989). Bacteria may also synthesize specific lactoferrin receptors to bind and extract iron from lactoferrin directly, as a method to adapt to lactoferrin reduced iron availability (Schryvers *et al.*, 1998). Either or both of these adaptations may help explain the lack of treatment effect on *Salmonella* in the current research.

A direct bactericidal activity independent of iron acquisition has been proposed for lactoferrin, in which the peptide lactoferricin is reported to have a broad antimicrobial activity against several gram negative bacteria (Wakabayashi *et al.*, 2003). Other reports (van der Strate *et al.*, 2001; Ajello *et al.*, 2002; Gomez *et al.*, 2003) suggest that lactoferrin contributes to the innate immune system of the host by interfering with microbial virulence (adhesion, internalization). Neutrophils provide a source of lactoferrin in external fluids (Masson *et al.*, 1969) in response to microbial challenge and are thought to augment the innate immune response against microbial infection at the mucosal surface. Determining whether or not lactoferrin produced this type of response in our experiments is difficult at best. It is unclear if the inoculated *Salmonella* (Exp. I) or the naturally-colonized *Salmonella* (Exp. II) infected the mucosal surface of the gastrointestinal tract or merely populated the luminal contents throughout. However, we would suspect that a lactoferrin-response such as this would only be effective or measurable at much lower populations of *Salmonella*.

While the void of treatment differences in this research is disappointing, it would be premature to dismiss lactoferrin as a potential pre-harvest intervention. It is likely that the large challenge dose used in this research simply overwhelmed any protective benefits offered by the lactoferrin. Future research should examine the protective effects of feeding lactoferrin to recently weaned pigs prior to *Salmonella* challenge, either administered in a lower oral dose or via exposure to *Salmonella*-positive pigs.

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REFERENCES

Ajello, M., R. Greco, F. Giansanti, M.T. Massucci, G. Antonini, and P. Valenti. 2002. Anti-invasive activity of bovine lactoferrin towards group A streptococci. Biochem. Cell Biol. 80:119-124.

Bessler, H.C., I.R. de Oliveira, and L.G. Giugliano. 2006. Human milk glycoproteins inhibit the adherence of *Salmonella typhimurium* to HeLa cells. Microbiol. Immunol. 50:877-882.

Blecha, F., D.S. Pollmann, and D.A. Nichols. 1983. Weaning pigs at an early age decreases cellular immunity. J. Anim. Sci. 56:396-400.

Brock, J.H. 1980. Lactoferrin in human milk: its role in iron absorption and protection against enteric infection in the newborn infant. Arch. Dis. Child. 55:417-421.

Crosa, J.H. 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. 53:517-530.

Di Mario, F., G. Aragona, N. Dal Bo, G.M. Cavestro, L. Cavallaro, V. Iori, G. Comparato, G. Leandro, A. Pilotto, and A. Franze. 2003. Use of bovine lactoferrin for *Helicobacter pylori* eradication. Dig. Liver Dis. 35:706-710.

Ellison, R.T., III, T.J. Giehl, and F.M. LaForce. 1988. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect. Immun. 56:2774-2781.

Foley, S.L., and A.M. Lynne. 2008. Food animal-associated *Salmonella* challenges: Pathogenicity and antimicrobial resistance. J. Anim. Sci. 86(14 Suppl):E173-87.

Frenzen, P.D., J.C. Buzby, and T. Roberts. 1999. An updated estimate of the economic costs of human illness due to foodborne *Salmonella* in the United States. In: Proc. of the 3rd Int. Symp. on the Epidemiology and Control of *Salmonella* in Pork, Washington, D.C., p. 215-218.

Gislason, J., I. Suhasini, T.W. Hutchens, and B. Lonnerdal. 1993. Lactoferrin receptors in piglet small intestine: Lactoferrin binding properties, ontogeny, and regional distribution in the gastrointestinal tract. J. Nutr. Biochem. 4:528-533.

Gomez, H.F., T.J. Ochoa, L.G. Carlin, and T.G. Cleary. 2003. Human lactoferrin impairs virulence of *Shigella flexneri*. J. Infect. Dis. 187:87-95.

Lee, N.Y., K. Kawai, I. Nakamura, T. Tanaka, H. Kumura, and K. Shimazaki. 2004. Susceptibilities against bovine lactoferrin with microorganisms isolated from mastitic milk. J. Vet. Med. Sci. 66:1267-1269.

Lee, W.J., J.L. Farmer, M. Hilty, and Y.B. Kim. 1998. The protective effect of lactoferrin feeding against endotoxin lethal shock in germfree piglets. Infect. Immun. 66:1421-1426.

Lonnerdal, B., and S. Iyer. 1995. Lactoferrin: molecular structure and biological function. Annu. Rev. Nutr. 15:93-110.

Masson, P.L., J.F. Heremans, and E. Schonne. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. J. Exp. Med. 130:643-658.

Naikare, H., K. Palyada, R. Panciera, D. Marlow, and A. Stintzi. 2006. Major role for FeoB in *Campylobacter jejuni* ferrous iron acquistition, gut colonization, and intracellular survival. Infect. Immun. 74:5433-5444.

Naidu, S.S., U. Svensson, A.R. Kishore, and A.S. Naidu. 1993. Relationship between antibacterial activity and porin binding of lactoferrin in *Escherichia coli* and *Salmonella typhimurium*. Antimicrob.

Agents Chemother. 37:240-245.

Ochoa, T. J., and T. G. Cleary. 2009. Effect of lactoferrin on enteric pathogens. Biochemie 91:30-34.

Pakkanen, R., and J. Aalto. 1997. Growth factors and antimicrobial factors of bovine colostrum. Int. Dairy J. 7:285-297.

Petschow, B. W., R. D. Talbott, and R. P. Batema. 1999. Ability of lactoferrin to promote the growth of *Bifidobacterium* spp. *in vitro* is independent of receptor binding capacity and iron saturation level. J. Med. Microbiol. 48:541-549.

Ratledge, C., and L. G. Dover. 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54:1319-1323.

Sarelli, L., M. Heinonen, T. Johansson, K. Heinonen, and H. Saloniemi. 2003. Lactoferrin to prevent experimental *Escherichia coli* diarrhea in weaned pigs. Int. J. Appl. Res. Vet. Med. 1:303-310.

Schryvers, A. B., R. Bonnah, R. H. Yu, H. Wong, and M. Retzer. 1998. Bacterial lactoferrin receptors. Adv. Exp. Med. Biol. 443:123-133.

Sherman, M.P., S.H. Bennett, F.F. Hwang, and C. Yu. 2004. Neonatal small bowel epithelia: enhancing anti-bacterial defense with lactoferrin and Lactobacillus GG. Biometals 17:285-289.

Shields, R. G., Jr., K. E. Ekstrom, and D. C. Mahan. 1980. Effect of weaning age and feeding method on digestive enzyme development in swine from birth to ten weeks. J. Anim. Sci. 50:257-265.

Still, J., P. Delahaut, P. Coppe, A. Kaeckenbeeck, J.P. Perraudin. 1990. Treatment of induced enterotoxigenic colibacillosis (scours) in calves by the lactoperoxidase system and lactoferrin. Annals Vet. Res. 21:143-152.

Teraguchi, S., H. Wakabayashi, H. Kuwata, K. Yamauchi, and Y. Tamura. 2004. Protection against infections by oral lactoferrin: evaluation in animal models. Biometals 17:231-234.

Tomita, M., H. Wakabayashi, K. Yamauchi, S. Teraguchi, and H. Hayasawa. 2002. Bovine lactoferrin and lactoferricin derived from milk: production and applications. Biochem. Cell Biol. 80:109-112.

Valenti, P., and G. Antonini. 2005. Lactoferrin: an important host defence against microbial and viral attack. Cell. Mol. Life Sci. 62:2576-2587.

van der Strate, B. W., L. Beljaars, G. Molema, M. C. Harmsen, and D. K. Meijer. 2001. Antiviral activities of lactoferrin. Antiviral Res. 52:225-239.

Van Leeuwen, P., S.J. Oosting, J.M.V.M. Mouwen, and M.W.A. Verstegen. 2000. Effects of a lactoperoxidase system and lactoferrin, added to a milk replacer diet, on severity of diarrhea, intestinal morphology and microbiology of digesta and faeces in young calves. J. Animal Physiol Animal Nutr. 83:15-23.

Vorland, L.H. 1999. Lactoferrin: a multifunctional glycoprotein. APMIS 107:971-981.

Wakabayashi, H., M. Takase, and M. Tomita. 2003. Lactoferricin derived from milk protein lactoferrin. Curr. Pharm. Des. 9:1277-1287.

Wang, Y., T. Shan, Z. Xu, J. Liu, and J. Feng. 2006. Effect of lactoferrin on the growth performance, intestinal morphology, and expression of PR-39 and protegin-1 genes in weaned piglets. J. Anim. Sci. 84:2636-2641.

Weinberg., E.D. 1995. Acquisition of iron and other nutrients in vivo. In: Virulence Mechanisms of Bacterial Pathogens, 2nd ed., pp. 79-93, Roth, J. A., Bolin, C. A., Brogden, K. A., Minion, F. C. and Wannemuehler, M. I. *et al.*, (eds), American Society for Microbiology, Washington, D.C.

Weinberg, E. D. 2001. Human lactoferrin: a novel therapeutic with broad spectrum potential. J. Pharmac. Pharmacol. 53:1303-1310.